

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number  
**WO 01/25455 A3**

(51) International Patent Classification<sup>7</sup>: C12N 15/82,  
15/29

(21) International Application Number: PCT/CA00/01144

(22) International Filing Date: 2 October 2000 (02.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/157,129 4 October 1999 (04.10.1999) US

(71) Applicant (for all designated States except US): **MED-ICAGO INC.** [CA/CA]; 2480, rue Hochelaga, Sainte-Foy, Québec G1K 7P4 (CA).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **VÉZINA, Louis-Philippe** [CA/CA]; 206, Route 138, Neuville, Québec G0A 2R0 (CA). **D'AOUST, Marc-André** [CA/CA]; 939, avenue Maurèse, App. 2, Québec, Québec G1S 2W9 (CA).

(74) Agents: **COTE, France et al.**; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report

(88) Date of publication of the international search report:  
8 November 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROMOTER FOR REGULATING EXPRESSION OF FOREIGN GENES

(57) Abstract: The present invention relates to a promoter for regulating expression of foreign genes in transgenic organisms, which comprises a promoter having the identifying characteristics of a promoter having a sequence selected from the group consisting of sequences set forth in SEQ ID NOS: 1 to 3 and functional fragments or derivatives thereof, wherein said promoter is adapted to be operationally located with respect to said foreign gene for expression of said gene.

WO 01/25455 A3



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/01144

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N15/82 C12N15/29

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, STRAND

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KENG-HOCK PWEE ET AL: "THE PEA PLASTOCYANIN PROMOTER DIRECTS CELL-SPECIFIC BUT NOT FULL LIGHT-REGULATED EXPRESSION IN TRANSGENIC TOBACCO PLANTS" PLANT JOURNAL, GB, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 3, no. 3, 1993, pages 437-449, XP002032746 ISSN: 0960-7412 the whole document --- -/--	1-8

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

6 June 2001

Date of mailing of the international search report

15/06/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

Oderwald, H

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAST D I ET AL: "PLASTOCYANIN IS ENCODED BY A SINGLE-COPY GENE IN THE PEA HAPLOID GENOME" PLANT MOLECULAR BIOLOGY,NL,NIJHOFF PUBLISHERS, DORDRECHT, vol. 12, 1989, pages 655-666, XP002032748 ISSN: 0167-4412 the whole document	1-8
P,X	WO 00 56906 A (MERISTEM THERAPEUTICS ;RANCE IANN (FR); THEISEN MANFRED (FR); GRUB) 28 September 2000 (2000-09-28) the whole document	1-8

## INTERNATIONAL SEARCH REPORT

### Information on patent family members

In International Application No

PCT/CA 00/01144

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0056906 A	28-09-2000	FR 2791358 A AU 3185300 A	29-09-2000 09-10-2000

## **PROMOTER FOR REGULATING EXPRESSION OF FOREIGN GENES**

### **BACKGROUND OF THE INVENTION**

#### **(a) Field of the Invention**

- 5           The invention relates to a promoter for regulating expression of foreign genes in a transgenic organism, more specifically in a leaf-specific manner in transgenic plants.

#### **(b) Description of Prior Art**

- 10           Genetic transformation of microbes have been used for more than 15 years to produce useful recombinant molecules, and applications in the pharmaceutical, cosmaceutical and dermaceutical industries are being currently exploited. This technology has expanded from microbes to plants and animals in the last ten years with the development of techniques required to adapt this general concept to complex eukaryotic organisms. Basically a gene encoding for a protein of interest or a gene encoding for an enzyme responsible for a modification of a metabolic pathway that leads to a molecule of interest, is linked in an appropriate fashion to cis-and trans-acting regulatory sequences, and transferred to a target cell where it is incorporated in the molecular machinery (in a transitory or stable fashion). The transgenic cell, or a tissue or organism regenerated from the transgenic cell will then perform transcription and translation of the transgene and therefore be enabled to accumulate the protein of interest or to perform the new metabolic reaction through the activity of the enzyme of interest.

- 25           The emerging industry of molecular farming is one of the most promising industry of the coming century. Its promise is to provide safe and renewable molecule factories for the industry. Among the applications that are currently developed are the production of low-cost monoclonal antibodies for therapeutic and diagnostic uses, the production of unlimited amounts of hormones, cytokines and other bio-active molecules for the treatment of chronicle or lethal diseases, the production of bio-safe substitutes for various blood components, the production of unlimited amounts of processing enzymes for the food and pulp industry, the production of low-cost enzymes for waste treatments, and the production of safe bio-active molecules for the cosmetic industry.
- 35

Limitations to the application of this technology has often come from the inability of transgenic organisms to accumulate adequate amounts of the recombinant product, as a result of low transcription rates, improper splicing of the messenger, instability of the foreign mRNA, poor translation rates, hyper-susceptibility of the recombinant protein to the action of endogenous proteases or hyper-susceptibility of the recombinant organism to the foreign protein which result in improper and limited growth or in the worst cases, in strong deleterious effects to the host organism. Inadequacy of production level has a direct impact on the development of applications when profit margins are narrow, or when treatment and/or disposal of residual matter causes bio-safety or environmental problems. Improvement of the accumulation level of the desired recombinant product thus appears to be one critical factor that warrants commercialization of many applications of molecular farming.

Photosynthesis is a metabolic reaction of paramount importance in the living world. It is performed by most land plants and algae, and by some bacteria. This overall reaction involves a complex assembly of electron transfer proteins spatially arranged within the thylakoid membrane system located in the chloroplast of leaf cells. This electron transport chain is coupled at one end with the photosynthetic antennae, which comprise a variety of macro-molecules, including one molecule common to all photosynthetic organism, chlorophyll, and at the other end, to the enzymes involved in NADPH and ATP synthesis, and to the Calvin cycle, involved in coupling the release of energy from NADPH and ATP with the fixation of gaseous carbon dioxide into organic molecules. Among the proteins involved in the overall photosynthetic reaction, one, Ribulose biphosphate carboxylase (Rubisco), is the most abundant protein on earth.

Photosynthesis is thus what leaf cells are dedicated to perform, and there is an obvious interest to use promoters of genes involved in such prominent tissue-specific metabolic activity when building strong leaf-specific expression cassettes for applications in plant biotechnology.

Many of the peptidic constituents of the photosynthetic apparatus are encoded by genes present in the chloroplastic genome; as an example, the heavy subunit of Rubisco, which bears the catalytic sites for CO<sub>2</sub> fixation, is encoded by a chloroplastic gene. However, the small

subunit of this enzyme is encoded by a nuclear gene, and thus the Rubisco holo-protein is made of subunits encoded by two different genomes. For obvious reasons, there has been a great interest in trying to use Rubisco promoters to control transcription of transgenes in leaves of transgenic plants. The promoter has been extensively characterized and its use in expression vectors is protected by United States Patent No. 4,962,028.

It would be highly desirable to be provided with a promoter for regulating expression of foreign genes in a transgenic organism, more specifically in transgenic plants.

### **SUMMARY OF THE INVENTION**

One aim of the present invention is to provide with a promoter for regulating expression of foreign genes in a transgenic organism, more specifically in transgenic plants.

In accordance with one embodiment of the present invention, there is provided a promoter for regulating expression of foreign genes in transgenic organisms, which comprises a promoter having the identifying characteristics of a promoter having a sequence selected from the group consisting of sequences set forth in SEQ ID NOS:1 to 3 and functional fragments or derivatives thereof, wherein said promoter is adapted to be operationally located with respect to said foreign gene for expression of said gene.

The preferred promoter of the present invention has a sequence selected from the group consisting of sequences set forth in SEQ ID NOS:1 to 3.

Preferably, the organism is a plant.

Preferably, the promoter of the present invention may be modulated by the presence or absence of light.

The preferred plant is a dicot, a monocot or a gymnosperm.

In accordance with another embodiment of the present invention, there is provided a method of regulating expression of foreign genes in transgenic organisms, comprising the steps of:

a) preparing a transgenic organism using an expression construct consisting of at least a promoter of the present invention, and an

ORF of a gene, wherein said promoter is operationally located with respect to said gene for expression of said gene.

For the purpose of the present invention the following terms are defined below.

5           The expression "functional fragments or derivatives thereof" is intended to mean any derivative or fragment of sequences SEQ ID NOS:1-3 which allow for an equivalent level of expression of a foreign gene as the promoter of the present invention set forth in SEQ ID NOS:1-3.

10

### **DETAILED DESCRIPTION OF THE INVENTION**

Following is a detailed description of the method used to generate transgenic alfalfa lines that can be regulated in their expression of a reporter gene.

15           In this embodiment, a promoter having the sequence set forth in SEQ ID NOS:1-3 was then ligated to a reporter gene and a terminator, and this construct was inserted in suitable plant expression vectors for DNA bombardment onto alfalfa leaves and for *Agrobacterium* mediated DNA transfer as described by Desgagnés et al. (1995, *Plant Cell Tissue Organ*  
20 *Cult.* 42:129-140). These two DNA transfer methods were used to demonstrate that expression of the reporter gene can be modulated by light.

### **Materials and Methods**

25

#### **DNA sequencing**

DNA sequencing was performed as described by Sanger et al (1977, *P.N.A.S. USA*, 74:5643-5647).

30           The resulting promoters of the present invention have the sequence as set forth in SEQ ID NOS: 1 to 3.

#### **Construction of expression cassettes and vectors**

35           The cassettes for expression analysis using the GUS reporter gene were assembled as follows. A promoterless GUS cassette was digested from pBI101 with HindIII and EcoRI, and was inserted into the



HindIII and EcoRI sites of the pUC19 polycloning site. The resulting plasmid was named pBI201 and was used for further constructs. Various deletion fragments of pGPlas3-2 were transcriptionally and transitionally fused at the 5'terminus of the GUS reporter gene in pBI201 and these were used for transitory expression studies using DNA bombardment. Upon identification of the adequate deletion fragment, it was subcloned into a binary plant expression vector such as pBI101 (Clontech). These recombinant plasmids were used for stable integration through *A. tumefaciens* infection as described below.

#### ***Agrobacterium*-mediated DNA transfer and regeneration of transgenic lines**

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation as described in Khoudi et al (1999, *Biotechnol. Bioeng.*, 64:135-143). Selected *Agrobacterium* strains were then co-cultivated with leaf disks from genotype C5-1 for 4 days in the absence of selection pressure (kanamycin). Following this incubation period, leaf disks were washed and pampered, and then allowed to form calli onto medium B5H. Calli were then transferred for 21 days on SH medium for embryo induction and for 28 days on BOi2Y for embryo development. Torpedo-shaped embryos were removed from Boi2Y and placed on MS medium for regeneration. Kanamycin was present in all cultivation medium except for co-cultivation and regeneration on MS. This method is described in length in Desgagnés et al (1995, *Plant Cell Tissue Organ Cult.* 42:129-140). Rooted plantlets were grown to maturity in the greenhouse.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

**WHAT IS CLAIMED IS:**

1. A promoter for regulating expression of foreign genes in transgenic organisms, which comprises a promoter having the identifying characteristics of a promoter having a sequence selected from the group consisting of sequences set forth in SEQ ID NOS:1 to 3 and functional fragments or derivatives thereof, wherein said promoter is adapted to be operationally located with respect to said foreign gene for expression of said gene.
2. The promoter of claim 1, wherein said promoter is modulated for transcriptional expression of said gene by presence or absence of light
3. The promoter of claim 1, wherein the promoter has a sequence selected from the group consisting of sequences set forth in SEQ ID NOS:1 to 3.
4. The promoter of claim 1, wherein said organism is a plant.
5. The promoter of claim 4, wherein said plant is a dicot, a monocot or a gymnosperm.
6. A method of regulating expression of foreign genes in transgenic organisms, comprising the steps of:
  - a) preparing a transgenic organism using an expression construct consisting of at least a promoter of claim 1, and an ORF of a gene, wherein said promoter is operationally located with respect to said gene for expression of said gene.
7. The method of claim 6, wherein said organism is a plant.
8. The method of claim 7, wherein said plant is a dicot, a monocot or a gymnosperm.

## SEQUENCE LISTING

<110> VÉZINA, Louis-Philippe  
D'Aoust, Marc-André  
MEDICAGO Inc.

<120> PROMOTER FOR REGULATING EXPRESSION OF  
FOREIGN GENES

<130> 14149-4 "PCT"

<150> US 60/157,129  
<151> 1999-10-04

<160> 3

<170> FastSEQ for Windows Version 3.0

<210> 1  
<211> 1350  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Sequence to be used as a Promoter for regulating  
expression

<400> 1

cgacggcccg	ggctggtata	tttatatggt	gtcaataaac	tcaaaaacca	taaaagttta	60
agtttagcaag	tgtgtacatt	tttatgtgaa	caaaaatatt	cacctactac	tggtataaat	120
cattattaaa	cattagagta	aagaaatatg	gatgataaga	acaagagtag	tgatattttg	180
acaacaattt	tggtgcaaca	tttgagaaaa	ttttgtgtgt	ctctcttttc	attgggtcaaa	240
aacaatagag	agagaaaaag	gaagagggag	aataaaaaaca	taatgtgagt	atgagagaga	300
aagttgtaca	aaagttgtac	caaaatagtt	gtacaaatat	cattgaggaa	tttgacaaaa	360
gctacacaaa	taagggttaa	ttgctgtaaa	taaataagga	tgacgcatta	gagagatgta	420
ccattagaga	atTTTTggca	agtcattaaa	aagaaagaat	aaattatttt	taaaattaaa	480
agttgagtca	tttgattaaa	catgtgatta	tttaatgaat	tgatgaaaga	gttggattaa	540
agttgtatta	gtaattagaa	tttggtgtca	aatttaattt	gacatttgat	cttttcctat	600
atattgcccc	atagagtcag	ttaactcatt	tttatatttc	atagatcaaa	taagagaaat	660
aacggtatat	taatccctcc	aaaaaaaaaa	aacggtatat	ttactaaaaa	atctaagcca	720
cgtaggagga	taacatccaa	tccaaccaat	cacaacaatc	ctgatgagat	aaccactttt	780
aagcccacgc	actctgtggc	acatctacat	tatctaaatc	acacattctt	ccacacatct	840
gagccacaca	aaaaccaatc	cacatcttta	tcacccattc	tataaaaaat	cacactttgt	900
gagtctacac	tttgattccc	ttcaaacaca	tacaaagaga	agagactaat	taattaatta	960
atcatcttga	gagaaaaatg	ccaccgttac	ttccaccacc	gttgctattc	catcattcac	1020
aggccttaag	gcaaacgcaa	gcaaagttaa	tgccatagct	aagggtccaa	cttcaacttc	1080
tcaattgcca	aggctttgtg	tcagagcttc	cctcaaagac	tttggagtgt	ctgctgtgtg	1140
cactgctgca	agtgcattgt	tagctagcaa	tgcccttgca	gttgaagtgt	tgcttggtgc	1200
tagtgatggg	ggtttggctt	ttgttcCaaa	caatttcaca	gtgaacgctg	gagacaccat	1260
tacattcaag	aacaatgctg	gttttcctca	caacgttatc	ttcgatgaag	acgagattcc	1320
aagcgggggt	gatgctgcaa	tcgaattccc				1350

<210> 2  
 <211> 971  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Sequence to be used as a Promoter for regulating expression

<400> 2  
 cgggctggta tatttatatg ttgtcaaata actcaaaaac cataaaagtt taagtttagca 60  
 agtgtgtaca tttttatttg aacaaaaata ttcacctact actgttataa atcattatta 120  
 aacattagag taaagaaata tggatgataa gaacaagagt agtgatattt tgacaacaat 180  
 tttgttgcaa catttgagaa aattttgttg ttctctcttt tcattgggtca aaaacaatag 240  
 agagagaaaa aggaagaggg agaataaaaa cataatgtga gtatgagaga gaaagttgta 300  
 caaaagttgt accaaaatag ttgtacaaat atcattgagg aatttgacaa aagctacaca 360  
 aataagggtt aattgctgta aataaataag gatgacgcat tagagagatg taccattaga 420  
 gaatttttgg caagtcatta aaaagaaaga ataaattatt tttaaaatta aaagttgagt 480  
 catttgatta aacatgtgat tatttaataga attgatgaaa gagttggatt aaagttgtat 540  
 tagtaattag aatttgggtg caaatttaaat ttgacatttg atcttttctt atatattgcc 600  
 ccataagatc agttaactca tttttatatt tcatagatca aataagagaa ataacggtat 660  
 attaatecct ccaaaaaaaaa aaaacggtat atttactaaa aaatctaagc cacgtaggag 720  
 gataacatcc aatccaacca atcacaacaa tcctgatgag ataaccact ttaagcccac 780  
 gcactctgtg gcacatctac attatctaaa tcacacattc ttccacacat ctgagccaca 840  
 caaaaaccaa tccacatctt tatcacccat tctataaaaa atcacacttt gtgagtctac 900  
 actttgattc cttcaaaca catacaaaga gaagagacta attaattaat taatcatctt 960  
 gagagaaaat g 971

<210> 3  
 <211> 731  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Sequence to be used as a Promoter for regulating expression

<400> 3  
 agagagaaaa aggaagaggg agaataaaaa cataatgtga gtatgagaga gaaagttgta 60  
 caaaagttgt accaaaatag ttgtacaaat atcattgagg aatttgacaa aagctacaca 120  
 aataagggtt aattgctgta aataaataag gatgacgcat tagagagatg taccattaga 180  
 gaatttttgg caagtcatta aaaagaaaga ataaattatt tttaaaatta aaagttgagt 240  
 catttgatta aacatgtgat tatttaataga attgatgaaa gagttggatt aaagttgtat 300  
 tagtaattag aatttgggtg caaatttaaat ttgacatttg atcttttctt atatattgcc 360  
 ccataagatc agttaactca tttttatatt tcatagatca aataagagaa ataacggtat 420  
 attaatecct ccaaaaaaaaa aaaacggtat atttactaaa aaatctaagc cacgtaggag 480  
 gataacatcc aatccaacca atcacaacaa tcctgatgag ataaccact ttaagcccac 540  
 gcactctgtg gcacatctac attatctaaa tcacacattc ttccacacat ctgagccaca 600  
 caaaaaccaa tccacatctt tatcacccat tctataaaaa atcacacttt gtgagtctac 660  
 actttgattc cttcaaaca catacaaaga gaagagacta attaattaat taatcatctt 720  
 gagagaaaat g 731

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**